

**METHODS AND COMPOSITIONS TO INCREASE
PLANT RESISTANCE TO STRESS**

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BACKGROUND

- [0001]** Environmental factors such as drought, soil salinity, and unfavorable temperatures lead to plant stress and result in reduced plant productivity. Crop yield losses of major crops such as rice, wheat, and maize due to abiotic stresses represent a significant economic and political factor and contribute to food shortages in many countries. Stress tolerant plants need to be developed to overcome these limitations and increase plant productivity. Traditional breeding strategies that attempt to select for genes that increase stress resistance have met with limited success because stress tolerance traits have complex inheritance making improvements by plant breeding schemes slow and unpredictable.
- [0002]** Most plants have evolved strategies to protect themselves against adverse environmental conditions such as drought and unfavorable temperatures. For example, when plants are exposed to unfavorable high growing temperatures, normal protein synthesis is reduced and rapid synthesis of heat shock proteins begins. Similarly, low temperature acclimation in plants is associated with the synthesis of hydrophilic proteins which act as cryoprotectants. Many plants respond to drought and salinity stress by accumulating high levels of proteins believed to protect plant tissues from osmotic stress. However, if the severity and duration of these stress conditions are intense or persist for a long time, the deleterious effects on plant development, growth and yield of most crop plants are significant. Continuous exposure to drought and high salt causes major alterations in the plant metabolism. These metabolic perturbations ultimately lead to cell death and consequently yield losses.
- [0003]** A generic stress signal transduction pathway is initiated by signal perception, followed by the generation of second messengers (*e.g.*, inositol phosphates and reactive oxygen species). The second messengers can modulate intracellular Ca^{2+} levels, often triggering a protein phosphorylation cascade that targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. The products of these genes may participate in the generation of regulatory molecules like plant hormones such as abscisic acid (ABA), ethylene,

and salicylic acid. These regulatory molecules can, in turn, propagate a second round of signaling response.

[0004] A plant hormone abscisic acid (ABA) mediates a variety of physiological processes, including the response to drought and salt stress. ABA is produced under water deficit conditions, which causes stomata closure and tolerance to drought and salt stress.

[0005] Altering the expression of certain transcription factors can greatly influence plant stress tolerance. For example, overexpression of the transcription factor C-BOX BINDING FACTOR-1 (CBF1) results in plants with increased tolerance to cold stress. Inducible expression of the transcription factor DEHYDRATION-RESPONSIVE ELEMENT BINDING-1A (DREB1A) also results in improved tolerance to several stress conditions, including drought, salt, and cold.

[0006] Stress signal transduction requires the proper spatial and temporal coordination of all related signaling molecules. MYB (originally identified as a transforming determinant in avian myeloblastosis virus) genes comprise a large family of transcription regulators in eukaryotes and are involved in a variety of biological functions. A common feature of MYB proteins is the presence of a functional DNA-binding domain (MYB domain) that is conserved among animals, plants and yeasts. The MYB domain typically consists of one to three imperfect (not identical) repeats, designated R1, R2 and R3. Each repeat is about 50 amino acids long and contains three helices, with the second and third helix forming the helix-turn-helix (HTH) DNA binding structure. MYB proteins show little sequence similarity outside the MYB domain and so are classified into subfamilies based on the number of repeats in the domain.

[0007] In plants, some MYB genes are characterized, and over 100 members have been identified in *Arabidopsis*. In contrast to animals, most plant MYB genes belong to the R2R3-MYB subfamily. Plant MYB genes have been shown to be involved in the regulation of many aspects of plant development, hormone signaling and metabolism. The “classical” MYB factors, which are related to c-Myb (cellular proliferation), may be involved in the control of the cell cycle in animals, plants and other higher eukaryotes. Functions of MYB proteins in plants include regulation of secondary metabolism, control of cellular morphogenesis and regulation of meristem formation and the cell cycle.

SUMMARY OF THE DISCLOSURE

[0008] Regulators of genes, especially transcription factors, involved in plant stress tolerance pathways are useful for engineering tolerance into plants because a single gene can activate a whole cascade of genes leading to the tolerant phenotype.

[0009] A method for improving response of a plant to stress includes the steps of:

- (a) transferring a DNA molecule, whose nucleotide sequence encodes a polypeptide that is at least 90% identical to an amino acid sequence as in SEQ ID NO: 1 to the plant; and
- (b) expressing the DNA molecule in the plant.

The DNA molecule includes a nucleotide sequence as in SEQ ID NO: 2, either stably integrated in the plant genome or transiently expressed. Other DNA molecules which also improve plant response to stress, may also be transferred to further improve response.

[00010] The stress may be cold, osmotic, drought, or abscisic acid.

[00011] A polypeptide is an *Arabidopsis thaliana* HOS10 transcription factor as in SEQ ID NO: 1. The plant is a monocot or a dicot.

[00012] A transgenic plant includes a recombinant nucleic acid encoding a HOS10 polypeptide (SEQ ID NO: 1), wherein increased expression of the protein within the plant results in increased cold resistance to the plant. The plant may be a monocot or dicot.

[00013] A plant seed includes a recombinant nucleic acid molecule encoding a polypeptide having an amino acid sequence that is at least 90% identical to SEQ ID NO: 1.

[00014] An expression cassette includes a promoter functional in a plant cell operably linked to an isolated nucleic acid sequence encoding an HOS10 polypeptide (SEQ ID NO:2), leading to an enhanced expression of the polypeptide in the plant cell results in increased cold resistance to the plant.

[00015] The promoter may be stress induced, e.g., an ABA-inducible promoter, a turgor-inducible promoter, and an ethylene responsive promoter.

[00016] The promoter may be a viral coat protein promoter, a plant tissue-specific promoter, a monocot promoter, a ubiquitin promoter, a CaMV 35S promoter, a CaMV 19S promoter, a *nos* promoter, an Adh promoter, a sucrose synthase promoter, a tubulin

promoter, a napin promoter, an actin promoter, a *cab* promoter, a PEP Case promoter, a 7S- α -conglycinin promoter, an R gene complex promoter, a tomato E8 promoter, a patatin promoter, a mannopine synthase promoter, a soybean seed protein glycinin promoter, a soybean vegetative storage protein promoter, a bacteriophage SP6 promoter, a bacteriophage T3 promoter, a bacteriophage T7 promoter, a P_{tac} promoter, and a root-cell promoter.

[00017] A plant vector includes a recombinant nucleic acid encoding a HOS10 polypeptide (SEQ ID NO: 1), wherein an expression of the polypeptide in a plant results in increased cold resistance of the plant.

[00018] A host plant cell includes a recombinant nucleic acid encoding a HOS10 polypeptide (SEQ ID NO: 1). Expression of the protein in the plant cells results in increased cold resistance of the plant.

[00019] A plant vector includes a recombinant nucleic acid encoding a HOS10 polypeptide (SEQ ID NO: 1). Expression of the polypeptide in a plant results in increased salt resistance of the plant.

[00020] A host plant cell includes a recombinant nucleic acid encoding a HOS10 protein (SEQ ID NO: 1). Expression of the polypeptide in a plant cell results in increased salt resistance of the plant.

[00021] A model plant used in the study of stress tolerance is *Arabidopsis thaliana*. A genetic screen that included a chimeric *RD29A::LUC* transgene and low light video imaging technology was used to isolate some *Arabidopsis* stress-responsive mutants. The *RD29A* promoter was cold-, osmotic stress- and ABA-responsive. Bioluminescent *Arabidopsis* plants expressing the *RD29A::LUC* transgene were used to identify some *Arabidopsis* mutants with altered induction of stress-responsive genes by high-salinity, cold and ABA. These mutants exhibited altered expression of the *RD29A::LUC* gene at a constitutive (*cos*), high (*hos*), or low (*los*) level in response to various abiotic-stress or ABA treatments.

Definitions

[00022] "promoter" refers to regions or sequences of a nucleic acid molecule located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Such a promoter can be derived from plant genes or from other organisms, such as viruses capable of infecting plant cells. A stress-inducible promoter refers to a

plant promoter capable of initiating transcription from an operably linked heterologous sequence in response to an exogenous stress response such as, for example, salinity, drought, and cold. The promoters need not be full length to function.

[00023] "operably linked" refers to functional linkage between a promoter and another nucleic acid molecule, wherein the promoter sequence initiates transcription of RNA corresponding to the other sequence.

[00024] "nucleic acid sequence" refers to an ordered set of deoxyribonucleotide or ribonucleotide bases forming a single or double-stranded molecule.

[00025] "plant" includes whole plants, shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

[00026] "recombinant" refers to a human manipulated polynucleotide or a copy or a complement of a human manipulated polynucleotide. For instance, a recombinant expression cassette comprising a promoter operably linked to another polynucleotide may include a promoter that is heterologous to the other polynucleotide as the result of human manipulation.

[00027] "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

[00028] "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences refer to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not

change the functional properties of the molecule. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity".

[00029] As used herein, *hos10-1* (and *myb8-1*) refer to a mutant DNA molecule caused by T-DNA insertion. *hos10* (*myb8*) refers to the corresponding genes. HOS10 (MYB8) refers to the polypeptide encoded by the *hos10* (*atmyb*) gene. HOS10 is synonymous with and AtMYB8 or simply MYB8.

BRIEF DESCRIPTION OF THE DRAWINGS

[00030] FIG. 1 shows that polypeptide HOS10 negatively controls *RD29A::LUC* expression in response to either low temperature, the phytohormone abscisic acid (ABA), or osmotic stress. WT is on the left half of a vertical separation, HOS10-1 on the right. Mutant HOS10-1 plant cells express more luminescence. (A) shows comparative luminescence; (B) quantified luminescence.

[00031] FIG. 2 shows that *hos10-1* mutants are susceptible to freezing treatment: (A) shows relative plant growth; (B) statistical analyses of relative survival; (C) electrolyte leakage assay.

[00032] FIG. 3 *hos10-1* mutant seed germination is hypersensitive to ABA inhibition (left panel) and *hos10-1* mutant plants lose more water under water deficit conditions (right panel). Error bars represent standard deviation (n=5 for left panel; n=8 for the right panel).

[00033] FIG. 4 shows that (A) and (B) *hos10-1* mutant plants are hypersensitive to NaCl, (C) LiCl; and (D) the mutant seedlings accumulated more Na⁺; (E) shows little difference in K accumulation.

[00034] FIG. 5 (A-E) shows developmental defects of *hos10-1* plants. The right side of each photo are the mutants, on the left are the wild type plants.

[00035] FIG. 6 shows (A) the position of the of the insertion of the T-DNA at the *hos10-1* locus; (B) complementation of *hos10-1* mutant phenotype with the wild type *hos10* gene; and (C) sequence alignment comparing HOS10 (AtMYB8) in various plant species. HOS10 encodes a R2R3-type MYB transcription factor. The arrows represent the boundaries of the R2 and R3 domains.

[00036] FIG. 7 shows the amino acid (SEQ ID NO: 1) (A) and the nucleotide (SEQ ID NO: 2) (B) sequence of the AtMYB8 transcription factor (HOS10).

DETAILED DESCRIPTION OF THE DISCLOSURE

[00037] While the concepts of the present disclosure are illustrated and described in detail in the drawings and the description herein, such an illustration and description

are to be considered as exemplary and not restrictive in character, it being understood that only the illustrative embodiments are shown and described and that all changes and modifications that come within the spirit of the disclosure are desired to be protected.

[00038] HOS10 appears to be a negative regulator of stress-responsive genes including *RD29A*. A mutant, *hos10-1*, which shows elevated induction of the *RD29A::LUC* gene in response to low temperature, ABA or NaCl (FIG. 1), was selected for characterization. The genetic locus responsible for this mutation was identified by TAIL-PCR, and *hos10* encodes a R2R3-type MYB transcription factor, corresponding to AtMYB8 (GenBank Accession number: AF207991; gene identifier At5g35515). Transgenic plants that express *hos10* either constitutively or in response to a stress response such as drought (e.g., under an ABA-inducible promoter), show more resistance or tolerance towards the external stress than a plant that does not express *hos10*, or expresses it at a lower level.

[00039] FIG. 1 shows that *RD29A::LUC* expression is significantly higher in *hos10-1* than in the wild type seedlings in response to low temperature, ABA, and NaCl. The expression of *RD29A::LUC* is higher in *hos10-1* than wild type even under room temperature. The *hos10-1* mutation enhances the *RD29A::LUC* expression in response to cold, ABA or osmotic stress. (A) *RD29A::LUC* expression was quantitatively measured as luminescence intensity (counts/plant). Shown are wild type and *hos10-1* seedlings and their luminescence images taken at room temperature, or after treatment at 0°C for 24h, or with 100 M ABA for 3h or 300 mM NaCl for 5h. The scale bar at the right shows the luminescence intensity from dark (lowest) to white (highest). (B) Quantification of luminescence images as shown in (A). Data represent the averages of luminescence intensities from 20 individual seedlings. Error bars indicate the standard deviation.

[00040] Because *hos10-1* mutant plants display enhanced sensitivity to cold, salinity, and ABA treatments, expression of HOS10 (MYB8) results in enhanced resistance or tolerance to these stress responses. For example, ectopic expression of HOS10 polypeptide in *hos10-1* mutant plants resulted in increased resistance to cold treatment compared to *hos10-1* mutant plants transformed with an empty vector (Example 1).

[00041] In freezing tolerance assays, *hos10-1* mutant plants appeared dramatically more sensitive to freezing (FIG. 2). *hos10-1* mutant plants were defective in cold acclimation (FIG. 2), leading to freezing sensitive phenotype. (A) Four-week-old

wild type and *hos10-1* plants grown in a growth chamber were cold-acclimated at 4° C under light (16h light, 8h dark) for 8d before freezing treatment (-8° C for 4h). The photograph was taken 7d after the freezing treatment. (B) Statistical analysis of the plant survival 7d after the freezing treatment as shown in (A) The error bars indicate standard deviation (n=60). (C) Electrolyte leakage assay. Cold-induced acclimation was obtained by exposing plants at 4° C for 8d. *hos10-1* (A), *hos10-1* cold acclimated; wild type (A), wild type with cold acclimation. Error bars represent standard deviation (n=8)

[00042] Expression of *myb8 (hos10)* in a tissue specific manner is also within the scope of this disclosure. For example, expression of *myb8 (hos10)* under a root-specific promoter results in the selective expression of *myb8 (hos10)* in plant roots and offers protection to root cells from high salinity or freezing. A root specific promoter includes for example a promoter for metallothionein-like gene PsMTA from pea (Fordam-Skelton, *et al.* 1997). Other examples of cis acting plant regulatory sequences for plant promoters can be found in <http://rarge.gsc.riken.go.jp/cdna/promoter/> and Higo *et al.* (1999).

[00043] Tissue specific expression of *myb8 (hos10)* in flowers or any other floral parts results in increased tolerance to freezing. For example, expression under control of a petal or sepal specific promoter leads to freezing tolerance during adverse temperatures. A chalcone synthase (CHS) promoter from petunia is an example of a petal specific promoter.

[00044] Tissue specific expression of *myb8 (hos10)* in fruits, leaves, stems, petioles or any other plant part results in increased tolerance to freezing.

[00045] The observed higher induction of *RD29A* in the *hos10-1* mutant plants may be due to a feedback response due to hypersensitivity. Because the mutant is highly sensitive to stress treatments and stress-responsive genes such as *RD29A* are more easily activated to compensate for the reduced stress tolerance of the mutant. *HOS10* is likely important both for stress tolerance and stress gene expression.

[00046] The *hos10-1* mutation appears to affect the ABA sensitivity and the water loss. (FIG. 3). *hos10-1* mutant seed germination is hypersensitive to ABA inhibition (left panel) and *hos10-1* mutant plants lose more water under water deficit conditions (right panel). Error bars represent standard deviation (n=5 for left panel; n=8 for the right panel).

- [00047]** The *hos10-1* mutation appeared to affect the salt sensitivity of the mutant plants (FIG. 3). *hos10-1* plants are hypersensitive to NaCl and LiCl as shown in (A) *hos10-1* and wild type seedlings grown on MS medium or MS medium supplemented with 80 mM NaCl; (B) dosage response of *hos10-1* and wild type seedlings to NaCl. Data shown are relative root growth (growth on 0 mM NaCl was considered 100%); (C) dosage response of *hos10-1* and wild type seedlings to LiCl; Error bars in (B) and (C) indicate standard deviation (n=16). (D) Na⁺ accumulation in *hos10-1* and wild type seedlings; Error bars indicate standard error (n=8); (E) K⁺ accumulation in *hos10-1* and wild type seedlings. Error bars indicate standard error (n=8).
- [00048]** The *hos-10* locus was identified by TAIL-PCR and it encodes a R2R3-type MYB transcription factor. The wild type *atmyb8* gene was able to complement the *hos10-1* mutant (FIG. 5).
- [00049]** Expression of *atmyb8* (*hos10*) in *hos10-1/myb8-1* mutant plants restored tolerance to freezing (FIG. 6). The structure of *hos10* gene and the position of the T-DNA insertion relative to the translation initiation codon which is the mutant form is shown in FIG. 6 (A). Filled boxes indicate exons; lines indicate introns. A T-DNA inserted in the *hos10-1/myb8-1* mutant is indicated; (B) shows that the genomic fragment containing the putative *hos10* gene complemented the *hos10-1/myb8-1* mutant. The photograph was taken 8d after the freezing treatment (-8°C for 3h). *myb8-1*+vector, *myb8-1* transformed with the empty vector which was used for complementation); *myb8-1*+MYB8, *myb8-1* transformed with a wild type *atmyb8* gene); (C) sequence alignment comparing *hos10* (*atmyb8*) with sequences from other species.
- [00050]** Depending upon the site of integration in the chromosome and the number of copies integrated, a transgenic plant may express the transgene to varying levels either constitutively or in response to a stimuli. Because the integration of a transgene is random, transgenic plants may have varying levels of expression. Standard RT-PCR or any other quantitative technique can be used to obtain a stably transformed transgenic plant selecting for desired expression levels.
- [00051]** Drought and low growth temperatures stresses may be governed by genes from multiple loci. For example, the cis-acting element identified in the promoter region of the RD29A gene, is responsive to both drought and low temperatures. This dehydration-responsive element (DRE) is required for the regulation of expression of dehydration responsive genes. CBF genes encode transcriptional activators that

control the expression of a number of genes containing C-repeat/DRE sequences in their promoters. Other transcriptional regulators, such as the MYC and MYB proteins, are activators in the dehydration and abscisic acid (ABA)-inducible expression of the *rd22* gene. The promoter of *rd22* gene contains a 67-bp fragment with two closely situated recognition sites for the basic helix-loop-helix protein MYC. There is also a putative recognition site for MYB in the promoter of the *rd22* gene. All three recognition sites function as cis-acting elements in the dehydration-induced expression of *rd22*. Thus, it is contemplated to express a plurality of genes in plants, whose expression are regulated or triggered by a plurality of signaling pathways.

[00052] A suitable promoter is any one of the following; or any promoter that functions in a plant to allow expression of the *hos10* gene. A viral coat protein promoter, a tissue-specific promoter, a monocot promoter, a ubiquitin promoter, a CaMV 35S promoter, a CaMV 19S promoter, a *nos* promoter, an Adh promoter, a sucrose synthase promoter, a tubulin promoter, a napin promoter, an actin promoter, a *cab* promoter, a PEPCase promoter, a 7S- α' -conglycinin promoter, an R gene complex promoter, a tomato E8 promoter, a patatin promoter, a mannopine synthase promoter, a soybean seed protein glycinin promoter, a soybean vegetative storage protein promoter, a bacteriophage SP6 promoter, a bacteriophage T3 promoter, a bacteriophage T7 promoter, a P_M promoter, a root-cell promoter, an ABA-inducible promoter or a turgor-inducible promoter, and any other stress inducible promoters. Stress inducible promoter are particularly helpful. Exemplary transiently activated stress-inducible plant promoters are described in U.S. Patent No. 6,414,221.

[00053] A plant vector includes an isolated nucleic acid encoding an HOS10 (AtMYB8) protein. Expression of the protein in a plant cell imparts increased cold, drought, and salt resistance to the plant cell. The plant vector can further include a promoter functional in a plant cell.

[00054] A transgenic plant includes an isolated nucleic acid (SEQ ID NO: 2) encoding an HOS10 protein. Increased expression of the protein within a plant cell over its background level imparts increased cold, drought, and salt resistance to the plant cell.

[00055] Transgenic plants of the disclosure can be a dicot or a monocot. Examples of monocot plants are, maize, rice, rye, millet, wheat, barley, sorghum, oats, bananas, and orchids. Examples of dicot plants are *Arabidopsis*, cabbage, potato, tomato, tobacco, oranges and other citrus fruits, squashes, cotton, tea, soybeans and all the other legumes cashews, pistachios, apples, peaches, strawberries.

[00056] Signaling pathways are conserved in monocots and dicots. Several key signaling pathways are conserved among dicots and monocots. For example, mitogen activated protein kinase (MAPK) cascades in stress response, are conserved in monocots and dicots. In addition, MYB8 (HOS10) homologs from *Arabidopsis*, tomato, tobacco, and rice show significant structural similarity (FIG. 6C). Signaling mechanisms mediated by plant hormones such as abscisic acid (ABA) and ethylene are also conserved among plant species. Several genes from dicots such as *Arabidopsis* have been functionally expressed in monocots such as rice. Transgenic expression of MYB8 (HOS10) either constitutively or under an inducible or a tissue specific promoter results in increased resistance to stress tolerance in both monocots and dicots.

[00057] A seed includes an isolated nucleic acid encoding a HOS10 polypeptide. Expression of the polypeptide within a plant cell imparts increased cold, drought, and salt resistance to the plant cell (SEQ ID NO: 1).

EXAMPLES

Example 1: Expression of HOS10 in *hos10-1* mutant plants complements sensitivity to stress

[00058] The *hos10-1/myb8-1* mutant plants were transformed with an expression vector containing a wild-type *hos10* gene under the control of a CaMV 35S promoter using standard transformation techniques. FIG. 6 (B) shows that the genomic fragment containing the putative *hos10* gene complemented the *hos10-1/myb8-1* mutant. The photograph was taken 8d after the freezing treatment (-8°C for 3h) The treatment samples were *myb8-1*+vector, *myb8-1* transformed with the empty vector (used for complementation); *myb8-1+myb8*, *myb8-1* transformed with a wild type *hos10* gene. The *hos10-1* mutant transformed with a wild-type *hos10* gene under the control of a CaMV 35S promoter were not susceptible to freezing conditions.

Example 2: Overexpression of HOS10 Increases Plant Tolerance to Cold Temperature

[00059] Plants transformed with the vector containing only ³⁵S promoter (6), nontransformed (7) and ³⁵S promoter driving the *hos10* gene (1-5) were acclimated to cold (4°C) for 1 week. Plants were subsequently exposed to freezing temperatures 0°C to -16°C for 24 hr. The lowest temperature at which all of the plants survived was recorded and given as the cold tolerance of each transgenic line. Expression of

the HOS10 gene was estimated relative to control (transformed with vector only) by RT-PCR of the HOS10 coding sequence cDNA.

TABLE 1: Overexpression of HOS10 Increases Plant Tolerance to Cold Temperatures

Construct	Transgenic line No.	Expression of HOS10	Cold tolerance phenotype (minimum temperature for survival °C)
³⁵ S promoter:HOS10	1	++++	-14
³⁵ S promoter:HOS10	2	+++	-11
³⁵ S promoter:HOS10	3	+++	-12
³⁵ S promoter:HOS10	4	+++	-12
³⁵ S promoter:HOS10	5	++++	-13
³⁵ S promoter only	6	+	-9
nontransformed	7	+	-9

The results of RT-PCR experiments (TABLE 1) show relative absorbance units of plants expressing HOS10 under a 35S promoter when compared to nontransformed plants. For example, absorbance units of a nontransformed plant (line no. 7; TABLE 1), was used as basal value (+) to calculate the relative expression of HOS10 in HOS10 overexpressing plants.

MATERIALS AND METHODS

Transformation

[00060] A DNA segment identified in this disclosure is isolated, and combined with at least a promoter functional in a plant cell to provide a recombinant expression cassette. An expression cassette comprising a recombinant DNA segment is subcloned into any standard expression vector by methods known to those of skill in the art. Suitable expression vectors include plasmids that autonomously replicate in prokaryotic and/or eukaryotic cells. The expression vector is introduced into prokaryotic or eukaryotic cells by currently available methods such as, protoplast transformation, *Agrobacterium*-mediated transformation, electroporation, microprojectile bombardment, tungsten whiskers and liposomes. The vector is introduced into prokaryotic cells such as *E. coli* or *Agrobacterium*. Transformed cells is selected typically using a selectable or screenable marker encoded on the expression vector. A method to generate transgenic monocot plants is described in U.S. Patent No. 6,281,411, the disclosure of which is herein incorporated by reference as a method.

[00061] The expression cassette or vector is introduced into plant cells that include both monocot and dicot plants. Plant cells, or tissue, or organs useful for transformation include flowers, callus, immature embryos, meristematic tissue, gametic tissue, or cultured suspension cells. Other recombinant DNA molecules encoding proteins that enhance plant transformation may also be introduced. The transformed plant cell is regenerated into a plant and the plant tested for its ability to grow or thrive under stress conditions, such as high salinity, reduced water availability or adverse temperatures. Depending on the type of plant, the level of gene expression, and the activity of the protein encoded by the recombinant DNA segment, introduction of the recombinant DNA into the plant confers the phenotype of tolerance or resistance to the stress conditions to the plant.

[00062] The introduced recombinant DNA segments is expressed in the transformed plant cells and stably transmitted (somatically and sexually) to the subsequent cells produced. The vector is capable of introducing, maintaining, and expressing a recombinant DNA segment in plant cells, wherein the recombinant DNA is obtained from a variety of sources, including to plants and animals, bacteria, fungi, yeast or virus. Additionally, the vector is introduced into a wide variety of cells of plants.

[00063] Introduction and expression of foreign genes in dicotyledonous plants such as *Arabidopsis*, tobacco, potato and alfalfa has been accomplished using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. Through recombinant DNA techniques, a wide variety of foreign DNAs can be inserted into the T-DNA in *Agrobacterium*. Following infection by the bacterium containing the recombinant Ti plasmid, the foreign DNA is inserted into the host plant chromosomes, thus producing a genetically engineered cell and then a genetically engineered plant. Another approach is to introduce root-inducing (Ri) plasmids as gene vectors. Additionally, using the Ti plasmid as a model system, it may be possible to artificially construct transformation vectors for monocot plants. Ti-plasmids may also be introduced into monocots by artificial methods such as microinjection, or fusion between monocot protoplasts and bacterial spheroplasts containing the T-region, which can then be integrated into the plant nuclear DNA. The selectable-marker and heterologous-gene expression cassettes described herein may be placed in a suitable expression vector designed for operation in plants. Suitable vectors are described in, for example, U.S. Pat. Nos. 5,888,789 and 5,889,189.

[00064] Transformation of plant cells with a recombinant DNA segment may also be accomplished by introducing a recombinant DNA into other nucleic acid molecules that can transfer the inserted DNA into a plant genome, *e.g.*, plant pathogens such as DNA viruses like CaMV or geminiviruses, RNA viruses, and viroids; DNA molecules derived from unstable plant genome components like extrachromosomal DNA elements in organelles (*e.g.*, chloroplasts or mitochondria), or nuclear encoded controlling elements; DNA molecules from stable plant genome components (*e.g.*, origins of replication and other DNA sequences which allow introduced DNA to integrate into the organellar or nuclear genomes and to replicate normally, to autonomously replicate, to segregate normally during cell division and sexual reproduction of the plant and to be inherited in succeeding generations of plants) or transposons.

[00065] A recombinant DNA may be delivered into plant cells or tissues directly by microorganisms with infectious plasmids, infectious viruses, the use of liposomes, microinjection by mechanical or laser beam methods, by whole chromosomes or chromosome fragments, electroporation, and microprojectile bombardment. For example, callus cells or any other suitable tissue derived from the members of a desired plant species are transformed with an expression cassette that includes at least

the recombinant DNA segment of interest, a selectable marker, and a suitable promoter using a variety of standard techniques (e.g., electroporation, *Agrobacterium*, protoplast fusion, or microparticle bombardment). A suitable transcription regulatory region (promoter) includes a plant promoter whose transcription is regulated in response to various stress responses such as drought, salinity, and high temperature.

[00066] **Isolation of *hos10-1* mutant:** *Arabidopsis thaliana* plants (ecotype C24) expressing the homozygous transgene *RD29A::LUC* referred to as wild type (Ishitani *et al.*, 1997) were mutagenized with an *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation using the activation tagging vector pSKI015 (Zhu *et al.*, 2002). Seeds from T₂ plants were used for screening mutants with altered *RD29A::LUC* expression in response to low temperature, ABA, and/or osmotic stress by luminescence imaging with a CCD camera as described by Ishitani *et al.* (1997).

[00067] **Freezing tolerance:** Wild type and *hos10-1* plants grown in soil at the rosette stage were used for the freezing tolerance test. For cold acclimation treatment, plants were placed in a cold room at 4°C under a long day photoperiod (16h light, 8h dark). Fully developed rosette leaves were used to determine freezing-caused electrolyte leakage essentially as described by Sukumaran and Weiser (1972) and Ristic and Ashworth (1993). Briefly, for each treatment, one excised leaflet was placed in a test tube containing 200 µl of HPLC grade H₂O, and the tubes were placed in a programmable cooling bath (model RTE-210; Thermo NesLab, Portsmouth, NH) with the temperature set at 0°C. There were 8 replicates for each temperature treatment. The temperature of the bath was programmed to decrease to -10°C, with 1°C decrement after 30 min. The tubes were removed from the bath when the designated temperature was reached, and they were placed immediately on ice water to allow gradual thawing for overnight. The tubes then were added with additional 5 ml HPLC H₂O and shaken for 24h, and the conductivity of the solution was measured with a conductance meter (model 35, YSI Co., Ohio). The tubes with the leaflets were then heated to boil for 40 min. After cooling down to room temperature, conductivities of the solutions were measured again. The percentage of electrolyte leakage was calculated as the percentage of the conductivity before boiling over that after boiling.

[00068] Three-week-old wild type and *hos10-1* plants grown in soil in a growth chamber were first incubated at 4°C under long day photoperiod for one week for cold acclimation. After this cold acclimation, the plants were subjected to freezing at -8°C

for 4h. The plants were transferred immediately to 4°C after the treatment is finished and incubated overnight to allow the frozen soil to thaw gradually. Plant damage was scored 7d later.

[00069] **Cloning of HOS10 gene:** DNA fragment flanking the left border of the inserted T-DNA in *hos10-1* plants was isolated by TAIL-PCR (Liu et al., 1995; Zhu et al., 2002) and subcloned into cloning vector pBluscript SK (+) (Stratagene, La Jolla, CA) as described (Zhu et al., 2002). The entire isolated fragment was sequenced. The following primer pair was designed to perform T-DNA diagnosis PCR (Zhu et al., 2002): forward 5'-ACAAATCGAGTCGGACTGTACC-3'; reverse, 5'-TCCATCGGCTTACTCTACGTCG-3'.

[00070] The coding region of HOS10 was amplified by *reverse transcription* (RT)-PCR. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) from wild type plants (ecotype Columbia) and 3 µg of total RNA was used for first-strand cDNA synthesis using thermoscript™ RT-PCR system (Invitrogen, Carlsbad, CA). The gene specific primers for HOS10 were as follows: forward, 5'-ACTGGAGCTCATGGGAAGATCACCATGTTGTG-3' (SacI site is underlined) and reverse, 5'-ACGTTCTAGACACACGAGCTAGTAACAAGATC-3' (XbaI site is underlined). The RT-PCR product was then subcloned into pGEM-T Easy Vector with the pGEM-T Easy Vector System (Promega, Madison, WI), resulting the cDNA clone10-133 and the sequence of the insert was confirmed by sequencing. The HOS10 was then released from clone 10-133 and cloned into binary vector 99-1 between SacI and XbaI sites, resulting the expression cassette of HOS10 under the CaM 35S promoter. The construct was introduced into *hos10-1* mutant plants through an *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation. Primary transformants, which were resistant to 50 mg/L hygromycin (Invitrogen), were transferred to soil to grow to maturity. Progenies of these transformants were examined for *RD29A::LUC* expression with the CCD camera and for freezing tolerance as described above.

[00071] **Ion content measurements and salt stress test:** Seeds were surface sterilized (2% sodium hypochloride for 15 min.), sowed onto medium containing Murashige and Skoog (MS) basal salt mixture, 2% sucrose solidified with 1% agar (pH 5.7). After sowing the seeds were stratified for 2 to 4 days at 4° C. For the mutant characterization *in vitro*, MS either contained no supplement or was supplemented

with 40, 80, 100, 120, 150, 180, 200 mM NaCl, or 5, 10, 15, 20, 25, 30 mM LiCl. Three-week-old seedlings (n=40-60) grown on germination medium (0.7% agar) were transferred to a 500 ml flask containing 200 ml 1/2-strength MS salts, 2 % sucrose (pH 5.7). The flasks were shaken at 120 rpm under cool-fluorescent light (16h light, 8h dark) at 22°C. After 2d, NaCl was added to give the desired final NaCl concentrations, and the seedlings were allowed to grow for additional 2d. The seedlings were then harvested, rinsed with excess amount of deionized H₂O, dried at 65°C for 48h and weighed. Ground tissues (300 mg each) were then extracted with 0.1 M nitric acid for 4h and filtered through filter paper (Fisher Scientific). Na⁺ or K⁺ content was determined by atomic absorption spectrometer (Model SpectrAA-10, Varian, Springvale, Australia).

[00072] **Seed germination and water loss:** Surface-sterilized *hos10-1* and wild type seeds were sown in Petri dishes on filter paper soaked with water or different concentrations of ABA. The Petri dishes were placed under light at room temperature for germination after being kept in darkness at 4°C for 3 days to break the dormancy. A seed was considered germinated when the radical emerged.

[00073] Detached shoots from soil-grown non-treated or ABA treated (100 µM ABA for 3h) *hos10-1* and wild type plants were placed on laboratory bench to lose water over a 3-hour time period. The water loss was expressed as the percentage of the original fresh weight.

[00074] DOCUMENTS CITED

The following documents are incorporated by reference to the extent they relate to protocols used in this disclosure.

Fordam-Skelton, *et al.* (1997) GUS expression in *Arabidopsis* directed by the 5' regions of the pea metallothionein-like gene PsMTA. *Plant Molecular Biology* 34: 659-668.

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Ristic and Ashworth (1993). Changes in leaf ultrastructure and carbohydrates in *Arabidopsis thaliana* L. (Heyn) cv. Columbia during rapid cold acclimation. *Protoplasma* 172: 111-123

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Zhu *et al.*, (2002) *Plant Cell* 14(12):3009-28